Conservation of two endangered European freshwater mussels (Bivalvia: Unionidae): A three-year, semi-natural breeding experiment

Rafael Araujo
Museo Nacional de Ciencias Naturales-CSIC
c/ José Gutiérrez Abascal 2
28006 Madrid, SPAIN
rafael@mncn.csic.es

Carles Feo
Quim Pou
Miquel Campos
Consorci de l'Estany
Plaça dels Estudis 2
17820 Banyoles, Girona, SPAIN
cfeo@consorcidelestany.org
qpou@consorcidelestany.org
mcampos@consorcidelestany.org

ABSTRACT

Freshwater mussels are among the most imperilled of all animal groups. The populations of the endangered *Unio mancus* Lamarek, 1819 and *U. ravoisieri* Deshayes, 1847 (both designated as *U. elongatulus* C. Pfeiffer, 1825 in the European Habitat Directive) have declined severely over recent years in Spain. To conserve these species in Lake Banyoles (Girona, Spain), a total of 108,875 *U. mancus* and 27,423 *U. ravoisieri* juveniles produced by artificial infection of larvae on host fish were grown in a number of semi-natural, sequential breeding systems, which involved the use of water and sediment from their natural habitat, plus pools, plastic outdoor channels, and/or cages. Across the tested systems, *U. mancus* reached a mean length of 9.7 mm (SD±1.53) in one year and 12.4 mm (SD±1.55) in two years; for *U. ravoisieri* these values were 15.8 (SD±0.76) and 21.2 mm (SD±2.45). In a experiment adding extra food, the growth rates were much lower than those recorded for the other systems. In October 2013, 278 2+ juveniles of *U. mancus* and 224 2+ juveniles *U. ravoisieri* were released into the lake, increasing their original populations by some 40% and 200% respectively. Preliminary observations made eight months later showed that several tens of these mussels were still alive. The large numbers of juveniles raised in the semi-natural systems will help conserve future generations for these bivalves in Lake Banyoles. Over the three years of the project, 3,510 fish infected with a total of some 500,000 glochidia of one or the other species were also released. After 1.5 years, hundreds of juveniles (13–35 mm) arising from this release were detected. This is the first time in Europe that thousands of juveniles of any endangered freshwater mussel species have been bred in captivity without the addition of extra nutrients, demonstrating the practicality of low-tech and economical approaches to mussel population restoration.

Additional Keywords: *Unio*, *Unio elongatulus*, *Unio mancus*, *Unio ravoisieri*, growth, juveniles, restocking, Lake Banyoles

INTRODUCTION

Freshwater mussels, or naiads (Order Unionida), are among the most imperilled of all animal groups. Their numbers have drastically declined due to pollution, habitat deterioration, and declining numbers of host fish (Lydeard et al., 2004; Strayer et al., 2004). More than half of the USA's near 300 species are now either extinct, endangered, or threatened, and in Europe the extinction rate for naiad populations is growing (Cuttelod et al., 2011). In the USA, this scenario has encouraged attempts to develop naiad captive breeding techniques—work that has inspired similar attempts in Europe. The first documented studies on naiad artificial reproduction and propagation were performed on commercial species in the USA (Lefevre and Curtis, 1912; Coker et al., 1921; Howard, 1922); nacre for buttons was of great economic importance in North America at the beginning of the 20th century (Anonymous, 1914; Claassen, 1994). In addition to providing an excellent compendium of the natural history of freshwater mussels, these pioneering papers summarized knowledge on mussel breeding and cultivation that is still useful today.

The reproductive strategy of freshwater mussels involves an obligatory parasitic stage, in which the larvae (glochidia) attach to the external surface of a suitable host and metamorphose into free-living juveniles (Lefevre and Curtis, 1912; Kat 1984; Wachtler et al., 2001; Araujo et al., 2002; Rogers-Lowery and Dimock Jr, 2006; Barnhart et al., 2008). This, of course, is a major problem in the development of controlled naiad breeding systems. Controlled breeding, of which the main objective is to obtain larger numbers of juveniles from fish infected with glochidia than would be naturally produced, can be carried out in captivity or semi-captivity. Juveniles then need to be grown before their introduction

into natural habitats where populations have been depleted or entirely lost.

Gum, Lange, and Geist (2011) recently published a critical reflection on some of the captive breeding techniques used in Europe and the USA, with emphasis on those for the freshwater pearl mussel *Margaritifera margaritifera* (Linnaeus, 1758). The information in the latter work, and in other seminal publications in the field (see below) was used to develop the successful semi-natural systems for rearing endangered freshwater mussels reported here.

Along with the process of fish infection, the provision of an adequate diet for juveniles is a key problem that must be solved. The success of Lefevre and Curtis (1912), Coker et al. (1921), and Howard (1922) in rearing juveniles of several species was dependent on the use of the water, food, and sediment present in the mussels' natural ecosystems. The idea that these elements were necessary was confirmed many years later in Europe by Hruska (1999), who hypothesized that the food required by *M. margaritifera* juveniles comes from a healthy rhizosphere. Eutrophication, contamination, and silting of the immediate environment was deemed responsible for the absence of available habitat, juvenile food and the recruitment of young mussels. The success of Hruska (1999), who grew juveniles larger than 5 cm, relied on river bank restoration and a semi-captive breeding system that provided for natural feeding. In Spain, Comas and Valls (2007) grew juvenile *Unio mancus* Lamarck, 1819 to reproductive age in a system involving minimum management that made use of natural water and sediment—but not from the river were the mussels normally lived—without any extra nutrients. However, this work was only published as an internal document of the Catalan Regional Government. Other authors have developed more controlled systems (with more or less success) inspired by systems used in marine bivalve aquaculture, providing extra food in the form of algae (Hudson and Isom, 1984; Gatenby et al., 1996; Gatenby et al., 1997; O'Beirn et al., 1998; Henley et al., 2001; Araujo et al., 2003; Gatenby et al., 2003; Beck and Neves, 2003; Liberty, 2004; Guyot, 2005; Jones et al., 2005; Barnhart, 2006; Kovitvadhi et al., 2006; Liberty et al., 2007; Eversole, 2008; Kovitvadhi et al., 2008). These more controlled systems have inspired the main cultivation programs for *M. margaritifera* in Europe (Gum et al., 2011; Eybe, et al., 2013).

Although the use of algae has sometimes been successful in the rearing of presumably healthy juveniles, Nichols and Garling (2000, 2002) report the main dietary source of carbon for naiads living in rivers and lakes to be bacterial. Algae do, however, appear to provide key nutrients such as vitamins and phytosterols. Much remains to be learned about the diet of juvenile naiads in natural environments and in captivity. As part of the LIFE 08 NAT/E/000078 "Estany Project" which is dedicated to restoring the native aquatic fauna of Lake Banyoles (Girona, Spain; a Natura 2000 site), semi-natural systems were developed to rear two endangered European species of mussel: *U. mancus* and *U. ravoisieri* Deshayes, 1847 (both designated as *U. elongatulus* C. Pfeiffer, 1825 under the Habitat Directive, the main European law for species conservation). The first of these species lives in Spanish and French Mediterranean rivers; the limit of its eastward range, however, remains unknown (Araujo et al., 2009a; Prié and Puillandre, 2013). It is considered "near threatened" by the IUCN (Cuttelod et al., 2011). The second species, *U. ravoisieri*, is restricted to just two localities in Spain (Araujo et al., 2009a; Khalloufi et al., 2011). The Lake Banyoles populations of both species have been in severe decline in recent years, a consequence of the proliferation of invasive predatory fish. The five native fish species (*Anguilla anguilla, Gasterosteus aculeatus, Barbus meridionalis, Squalius laietanus and Salaria fluviatilis*) have been partially eliminated and replaced by the exotic *Micropterus salmoides, Lepomis gibbosus, Cyprinus carpio, Perca fluviatilis*, and *Sander luciperca* (Moreno-Amich et al., 2006).

It was hypothesized that cultivation systems connected to the natural habitat of these naiads would provide the unknown natural food required by the juveniles. This paper presents the first large-scale attempt to raise juveniles of endangered naiad species in Europe, using water and sediment from the mussels' natural environment.

MATERIALS AND METHODS

The work was performed at the Naiad Breeding Laboratory at Banyoles (Girona, Spain). This field station is located 500 m from Lake Banyoles (which lies in the Ter Basin) and receives a constant supply of lake water. Although in preliminary work several fish species were tested (including *Salaria fluviatilis, Luciobarbus graellsii, Phoxinus phoxinus*, and *Tinca tinca*) as hosts for the mussel larvae, *Barbus meridionalis* Risso, 1827 and *Squalius laietanus* Doadrio, Kottelat and Sostoa, 2007 were chosen since these species are native to the lake. The two naiad species raised were *U. mancus* and *U. ravoisieri*, both of which are native to the lake basin. The number of specimens of fish and naiads involved differed over the three years of the project (2010–2013) (Table 1).

The fish used as hosts were collected from the Rivers Ter, Terri, Brugent, Llémena and Osor (all in the Ter Basin), 1–4 weeks before infection with glochidia. Following capture these fish were maintained in outdoor pools (1,600 L) that received a flow of lake water.

The gravid naiads used came from the lake (*U. ravoisieri*) or its effluents (*U. mancus*); these were collected over the spring (the water temperature of the lake was monitored six times per day using a submerged thermometer (Thermotronic Getech Innova) to determine the water temperature suitable for the reproductive cycle to begin). These mussels were maintained in indoor aquaria for the collection of released glochidia (these remained viable for 48–72 h); they were then returned to their natural habitat. Mature glochidia were collected with pipettes and placed in aerated water for 5 min in a plastic Tupperware vessel (500 ml) containing a single fish. Infected fish were then

Table 1. Fish and naiad specimens used.

		U. mancus				*U. ravoisieri*			
Barbus meridionalis	TOTAL	2011	2012	2013	Total	2011	2012	2013	Total
Infected	3412	188	705	1038	1931	51	232	1198	1481
Released to the lake	2507		436	839	1275		150	1082	1232
Infected at the lab	905	188	269	199	656	51	82	116	249
Survivors at the lab	637	67	255	174	496	18	56	67	141
% survivors	70.3	35.6	94.8	87.4	75.6	35.3	68.2	58	56.6
Squalius laietanus									
Infected	1236	62	973	73	1108	46	18	64	128
Released to the lake	1003		871	73	944		0	59	59
Infected at the lab	233	62	102	0	164	46	18	5	69
Survivors at the lab	100	29	37	0	66	20	9	5	34
% survivors	42.9	46.8	36.6	100	40.2	43.5	50	100	49.2

kept in aerated conical tanks (180 L) at a temperature ranging between 15 and 22 °C; only fish infected over the same 3-day period were placed in the same tank. These tanks were equipped with biological and mechanical filters. The fish were provided feed every day until three days before the release of juveniles. The number of degree/days needed for metamorphosis to occur was based on Araujo et al. (2005) and Reis et al. (2013). The water in these tanks was filtered (filter mesh size 200 µm) and renewed three times per day.

Two days before juvenile release, a 200 µm mesh collector was installed in the water circuit to retrieve them. This collector was checked and juveniles collected once or twice daily until no more were found.

The juveniles obtained were observed under a binocular microscope to check their viability; those deemed alive were then assigned to either (Figure 1):

System 1) Plastic tray (240 cm long, 60 cm wide, 17 cm high) containing 5 cm-deep un-sieved lake sediment (water depth to sediment surface = 9 cm) supplied with a constant water flow. This system was maintained indoors under normal photoperiod conditions. Juveniles of both naiad species were placed together in this system. Survival was checked periodically and a random sample of specimens measured until the emptying of the system in October 2013, or:

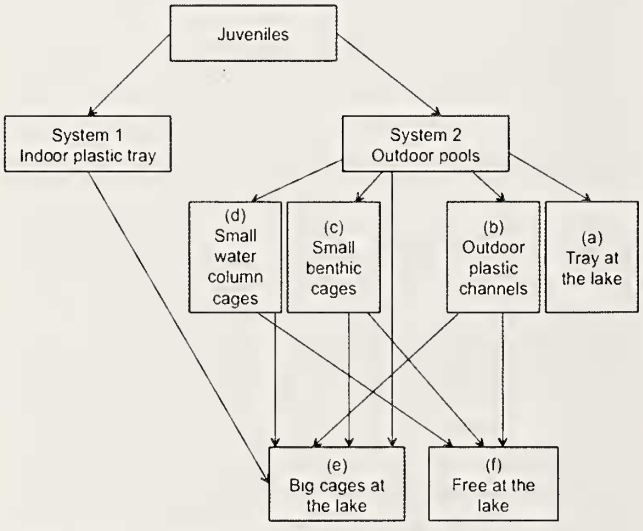

Figure 1. Schematic diagram of the experimental design.

System 2) Three outdoor cubic pools (150 × 150 × 150 cm) containing 20 cm-deep un-sieved lake sediment, (water depth to sediment surface = 110 cm) supplied with a constant water flow. *Unio mancus* juveniles were released into pool 1 and *U. ravoisieri* into pool 2 in 2011 and 2012; in 2012 the two species were also released together in pool 3. Survival was checked periodically and a random sample of specimens measured until pools 1 and 2 were emptied and the sediment filtered in February 2013, and until the same was performed with pool 3 in October 2013. [Note: new generations of *U. mancus* and *U. ravoisieri* are currently being raised in pools 1 and 2].

The surviving juveniles from System 2 were seeded into the following subsystems in February, April and October 2013 (Figure 1):

a) plastic tray containing lake sediment (depth 30 cm), placed at the bottom of the lake (depth 2 m).
b) outdoor plastic channels (6 m long, 50 cm wide, 28 cm high) containing 10 cm-deep lake sediment, supplied with a 1 L/s constant water flow (water velocity 50–100 cm/min) from the lake. Survival was checked periodically and a random sample of specimens measured each time until the end of the project.
c) plastic cages (30 × 15 × 15 cm; mesh 1 × 1 cm) placed on the lake bottom (depth 2 m) (only *U. mancus*).
d) cages (30 × 15 × 15 cm; mesh 1 × 1 cm) placed in the lake water column (depth 1 m) (only *U. mancus*).
e) cages (100 × 25 × 25 cm; mesh 1 × 1 cm) placed on the lake bottom (depth 2 m).
f) directly on the bottom of the lake in areas with no vegetation (only specimens produced in 2011 that reached a size of at least 2.5 cm).

Although the aim of the present work was to demonstrate the effectiveness of the natural diet (food from water and sediment) in raising the mussels, an experiment involving an external food source was also designed.

In 2012, 2000 *U. mancus* juveniles were divided into 5 series of 200 (two replicas) in Tupperware vessels

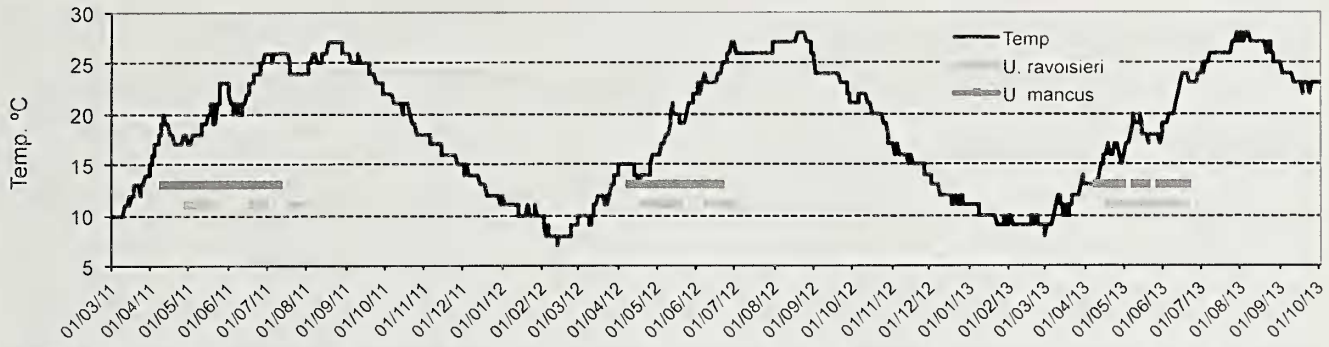

Figure 2. Lake temperature and glochidial release season for *Unio mancus* and *U. ravoisieri* over the three years of the experiment.

containing the following: 1) 400 ml lake water; 2) 400 ml lake water plus 0.8 ml of dehydrated commercial algae (66.5×10^9 cells of *Nannochloropsis*, 11.08×10^9 of *Phaeodactylum* and 1.25×10^9 of *Tetraselmis*); 3) 400 ml lake water plus a mixture of 2.4 ml of natural algae, leafs and macrophyte extract; 4) 400 ml lake water plus 0.8 ml of leaf extract and 0.8 ml of dehydrated commercial algae; and 5) 400 ml lake water plus 0.8 ml of biofilm extract.

Leaf extract was obtained by washing macrophytes and the leafs and stems of land plants from the habitat around the lake; the suspension obtained was filtered and frozen in doses of 0.8 ml. Biofilm extract was prepared from 200 g of the biofilm growing on the walls of the outdoor pools in 2 l of lake water; this was also filtered and frozen in doses of 0.8 ml.

All containers were cleaned once per week; dead juveniles were removed, live juveniles were measured, and the food renewed. The experiment ran between May 2012 and July 2013. The surviving juveniles were transferred into the lake in their own cage.

We performed a one-way ANOVA to compare the growth among some of the different systems.

RESULTS

In 2011–2013, the release of glochidia by *U. mancus* and *U. ravoisieri* occurred between April 11 and July 22, and April 27 and July 24, respectively (minimum lake temperature 13°C) (Figure 2). The total number of *U. mancus* and *U. ravoisieri* juveniles released by the host fish for use in the different systems was 108,875 and 27,423 respectively (Table 2). The release of juveniles from the host fish occurred between days 7 and 33 post infection (PI) in *U. mancus* and 8 and 26 PI in *U. ravoisieri*, depending on the water temperature (representing a minimum 145 and maximum 521 degree/days for both species taken together) (Table 3).

All the systems used in this study successfully raised mussels, but with marked differences in survival and growth rates. All the juveniles in the indoor plastic tray (System 1) in 2011 died due to a hardware malfunction, but in 2012 System 1 was capable of maintaining live juveniles (Figure 3). Those that survived one year (12%) reached a mean length of 4.5 mm (SD±1.35, n=102) and a maximum of 8 mm. The total survival rate at day 520 was 3.6%, with 574 live juveniles recovered (mean size 6.5 mm, minimum 3.5 mm, and maximum 11.5 mm) for the 16,322 originally seeded.

The best results were obtained with the outdoor pools (System 2). In 2011, 3,000 *U. mancus* juveniles were placed in pool 1 and about 1,800 *U. ravoisieri* in pool 2. In 2012 these figures were 9,380 *U. mancus* and 5,005 *U. ravoisieri* in pools 1 and 2 respectively. In addition, 16,658 *U. mancus* and 2,412 *U. ravoisieri* were mixed in pool 3. [Note: in 2013, once the juveniles from pools 1 and 2 had been removed and the pools cleaned, 36,140 *U. mancus* and 4,318 *U. ravoisieri* were placed in them respectively].

Table 2. Number of juveniles obtained from host fish, and numbers seeded in the different systems. Uma = *Unio mancus*. Ura = *U. ravoisieri*.

	2011		2012		2013		Total	
	Uma	Ura	Uma	Ura	Uma	Ura	Uma	Ura
Aquaria	329		489				**818**	**0**
Indoor plastic tray	7464	1592	15 447	875			**22 911**	**2467**
Outdoor pools	~3000	~1800	28 831	9719	36 140	4318	**67 971**	**15 837**
Outdoor channels					7766	9119	**7766**	**9119**
Tuppers			2653		601		**3254**	**0**
Lake			517				**517**	**0**
Lake streams and channels			5638				5638	**0**
TOTAL	**10 793**	**3392**	**53 575**	**10 594**	**44 507**	**13 437**	**108 875**	**27 423**

Table 3. Number of juveniles of *Unio ravoisieri* (above) and *U. mancus* (below) obtained per infection, fish species and year. B = *Barbus meridionalis*, S = *Squalius laietanus*.

	Month	Year	Fish	N fish	N juveniles	Temp	Days (max)	Degree/days (max)
U. ravoisieri	May	2011	S	5	1230	17.3	15–24 (19)	260–400 (330)
	April	2012	B	3	85	18.2	16–26 (22)	300–470 (400)
	May		B	7	5068	18.4	13–21 (19)	230–390 (350)
	July		B	6	278	21.4	11–17 (12)	235–365 (260)
	July		S	13	1216	21.4	11–17 (12)	235–365 (260)
	May	2013	B	18	2435	17.3	11–24 (19)	195–400 (330)
	May		B	30	3431	19.8	13–23 (15)	250–460 (290)
	June		S	8	336	22.0	8–15 (11)	175–325 (240)
	June		B	21	1677	22.6	8–15 (10)	175–340 (230)
	June		B	4	177	20.8	9–14 (12)	185–290 (250)
U. mancus	April	2011	B	7	838	17.0	17–23 (21)	285–390 (355)
	April		S	7	1279	17.1	17–26 (21)	290–445 (360)
	July		S	10	1774	20.9	10–17 (12)	215–355 (260)
	July		B	29	3610	21.0	10–17 (12)	210–360 (250)
	April	2012	S	18	5712	16.6	19–28 (21)	275–450 (325)
	April		S	9	294	15.1	19–26 (25)	280–390 (375)
	April		B	57	4075	16.3	18–33 (26)	232–521 (379)
	April		B	10	728	18.1	17–24 (19)	290–420 (325)
	May		B	82	35 356	18.8	12–25 (17)	190–465 (300)
	May		B	35	1156	19.2	14–22 (16)	260–425 (300)
	June		B	13	2551	21.5	10–19 (13)	200–400 (275)
	June		B	19	2731	21.5	7–19 (11)	145–410 (235)
	June		S	4	271	22.1	9–19 (13)	190–400 (275)
	April	2013	B	90	16 082	16.4	18–28 (23)	275–460 (364)
	April		B	10	2539	16.7	17–26 (21)	260–430 (335)
	April		B	54	21 987	16.8	17–25 (23)	345–437 (375)
	June		B	20	3825	21.0	9–17 (13)	190–360 (270)

Between summer and autumn 2012 there was a serious loss of juveniles in pool 1 probably due to the overgrowth of benthic algae; this problem was solved by cleaning the floor of the pool and covering the top. On day 350, 100 juveniles (≥1 cm) from pool 1 were transferred to a tray in the lake (System 2 a), and on day 575, 150 (1–1.5 cm) were transferred to the small cages described for System 2 c and d. On day 617, the pool was siphoned and a large number of empty shells detected. A total 493 remaining live juveniles were placed either in the outdoor plastic channels (System 2 b) or a large cage on the lake bottom (System 2e) (Figure 4A). On day 697, 100 juveniles (2–2.5 cm) from pool 2 were transferred to a large cage in the lake (System 2 e), and the remaining 237 distributed between the outdoor plastic channels (System 2 b) and a large cage in the lake (System 2 e) (Figure 4B). On day 325, 129 juveniles were transferred from pool 3 to a large cage in the lake (System 2 e). On day 500, the pool was siphoned and the 1,459 remaining juveniles detected transferred to another large cage in the lake (System 2 e).

The estimated survival rate at 1 year for the 2011 generation of *U. mancus* in pool 1 before any distribution into any subsystem was 77%. However, this fell to 20% after two years. The juveniles reached a mean length of 9.7 mm (SD±1.53, n=220 measured) after one year, and 12.4 mm

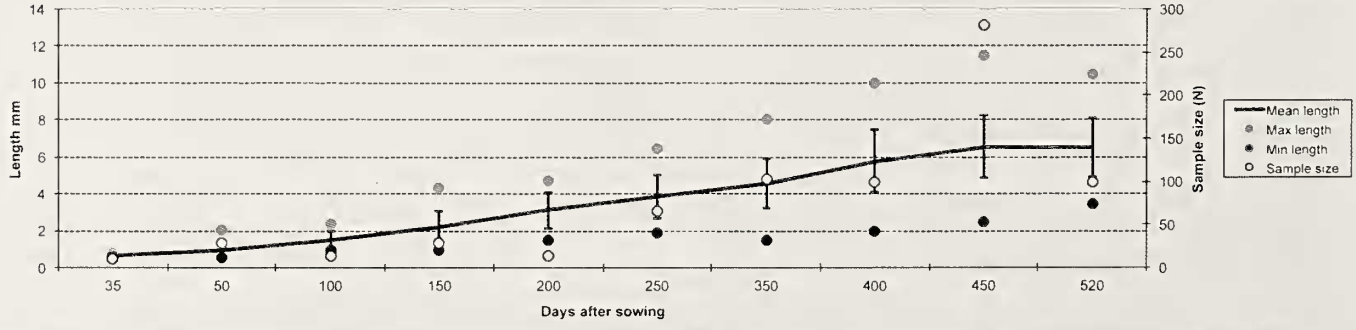

Figure 3. Growth of the juveniles seeded in 2012 in the indoor plastic tray (System 1) (both naiad species mixed). N indicates the number of measured juveniles.

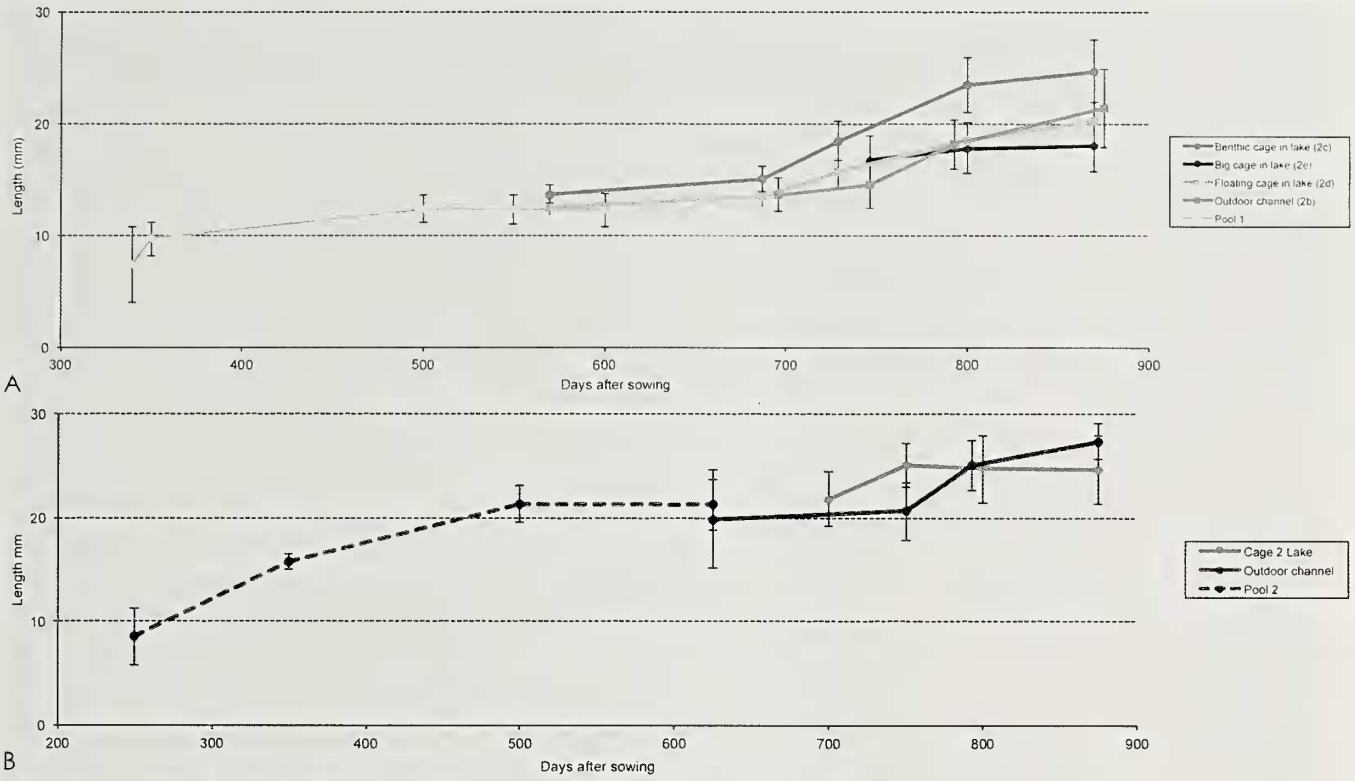

Figure 4. Juvenile growth in the different systems. A. *Unio mancus*. B. *U. ravoisieri*.

(SD±1.55, n=191 measured) after two (Figure 4A). The generation of juveniles seeded in 2012 were killed by the algal growth on the bottom.

In pool 2, the survival rate of the *U. ravoisieri* 2011 generation was 33% for the first year, and 18% for the second. The mean length reached at the end of the first and second years was 15.8 mm (SD±0.76, n=22 measured) and 21.2 mm (SD±2.45, n=331 measured) respectively (Figure 4B). The survival rate of the 2012 generation was 0.7%.

On day 500, the survival rate of the mixed population in pool 3 was 8.2% and the mean length 11 mm (SD±2.95, n=300 measured).

The survival and growth rates strongly increased when juveniles reached two years (2+) of age and a size of 1–1.5 cm in *U. mancus* (Figure 4A) and 2–2.5 cm in *U. ravoisieri* (Figure 4B). Of the 493 *U. mancus* 2+ juveniles in pool 1, 200 were transferred to a cage in the lake (System 2 e) and the rest to an outdoor plastic channel (System 2 b). After 170 days, the survival rates were 100% in the plastic channel and 93% in the cage, and the corresponding mean lengths were 2.1 (SD±2.33) and 1.8 cm (SD±2.3). In a similar experiment with *U. ravoisieri* 2+, the same survival rates were recorded but the mean lengths were much greater: 2.5 cm (SD±3.31) and 2.7 cm (SD±2.3) respectively. In October 2013 (i.e., at 870 days of age) a small portion of the juveniles in the outdoor plastic channels were maintained there; the rest were placed in the lake, both in cages (System 2 e) and free (System 2 f). In May 2012, prior to the use of the cages in the lake, 100 *U. mancus* juveniles of 1 cm from pool 1 were put in an open tray with sediment and placed in the lake (System 2 a). After three months the tray was removed; no living specimens were found but only broken shells, suggesting that they had fallen prey to fish and crayfish. However, in October 2012, upon inspection of the mud underneath where the cage had lain, a mussel was found measuring 28.1 mm (this mussel was marked for identification purposes), and in June 2013, another was found measuring 38 mm. This means that the first juvenile grew 17 mm in the five months since the cage was placed in the lake (May 2012) and the second 28 mm in 13 months. However, this subsystem was no longer used given the poor results obtained.

The survival rate at 295 days for the 150 *U. mancus* juveniles from pool 1 in the small cages in the water column and at the bottom of the lake (Systems 2 c and d), was very high at 83% and 86%, respectively. Growth, however, was greater in the cage on the bottom (System 2 c) ($F=93.7$, $p<0.001$) (Figures 4A, B). Indeed, the specimens in the water column cages were covered in algae and some showed deformities. The large, bottom-placed cages (System 2 e), though successful (Figures 4A, B) were difficult to handle. All the cages became covered in calcified algae during spring and summer, blocking the mesh and thus reducing oxygen and water flow. In October 2013, all the juveniles from all these cages were removed, labeled and placed once more in the lake, either in large cages (System 2 e) or free (System 2 f).

Over the three years of the project, 3,510 infected fish carrying an estimated total 500,000 *U. mancus* and *U. ravoisieri* glochidia were released into the lake and its effluents (Table 1). In May and July 2013 hundreds of 1.5 year-old (13–35 mm) juvenile mussels were observed in these outflows.

The results suggest that *U. mancus* reaches 9.7 mm by the end of its first year and 12.5 mm at the end of the second (taking all System 2 subsystems together and excluding System 1). For *U. ravoisieri*, these values are 15.8 and 21.2 mm. However, the growth rate is not constant over the year; growth stops between November and March (Figures 4A, B).

In the experiments involving the provision of extra food, one replica was followed for 400 days and the other 350. Although growth rates were reduced (maximum 2 mm in one year when provided with commercial algae and leafs; Figure 5) compared to the above tested systems, all survivors were placed in a large cage on the bottom of the lake (System 2e) in October 2013. At 350 days from the beginning of the experiment, only two of the five series had juveniles alive, both in the two replicas, the one with algae and the other with algae and leaf extract. The growth between the replicas didn't have significant differences ($p>0.02$), but it was different between the two series. The juveniles fed algae and leaf extract grew more than the others ($F=35.61$, $p>0.001$). However, the growth rates of the *U. mancus* juveniles of the experiment were less than the ones of the pool 1-system 2 ($F=1575.6$, $p>0.001$) or the plastic tray-system 1 ($F=271.9$, $p>0.001$).

In summary, the numbers of live juveniles (Figure 6) raised were: *U. mancus:* 218 2+ and 43,700 0+; *U. ravoisieri:* 100 2+, 64 1+ and 13,400 0+, plus a mixture (unknown proportions) of 2,304 1+ *U. mancus* and *U. ravoisieri* raised in pool 3.

A total of 278 and 224 2+ juveniles of *U. mancus* and *U. ravoisieri*, were released free into the lake, representing improvements of 40% (estimated population 1,000±500) and 200% (estimated population 110±50) of their original populations (M. Campos, pers. observ.).

DISCUSSION

Seminatural breeding efforts to rescue endangered pearl mussel populations can result in adverse effects such as genetic drift and selection (Geist, 2010), so they should only be considered as an emergency measure. The goal of this work was to obtain large numbers of juveniles of two endangered freshwater mussels, *U. mancus* and *U. ravoisieri*, raising them in a system involving water and sediment from their natural environment. The sequential systems tested maintained juveniles in pools or plastic channels at a field station, before releasing them into the wild. The large numbers of 1–3 years old juveniles maintained at the field station and in the lake offers hope for these endangered species. This is the first time in Europe that thousands of juveniles of any endangered freshwater mussel have been bred in captivity for three years without the addition of extra nutrients.

Although thousands of viable juveniles were raised, mortality was high, especially during the first year of life. The mortality recorded in the pools in 2012 was probably caused by algal overgrowth and subsequent anoxia. This can be avoided by covering the pool and/or siphoning and filtering the upper layer of the sediment, and renewing it after two years. The use of outdoor plastic channels with a slow water current—but fast enough to prevent deposition—can also be used. Although no natural mortality data are available for these species, juvenile mortality is commonly high among freshwater mussels (Young and Williams, 1984). The juveniles that reached two years seemed to show increased viability. The raising of mussels for one or two years in pools and then transferring the survivors to outdoor channels or bottom-lying cages in the wild, would likely provide very good results (Figures 4A, B).

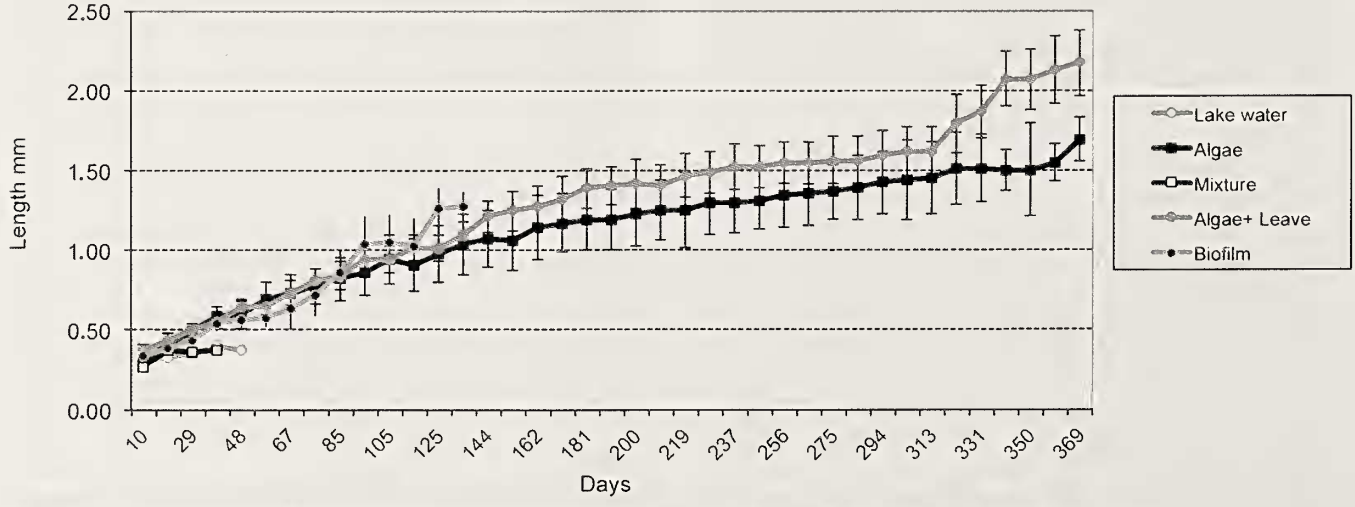

Figure 5. Survival and mean growth of the juveniles fed with extra food. Results are means for the two replicas.

Figure 6. The juveniles bred. A. Empty shells of *Unio mancus* and B. *U. ravoisieri* 2+ from System 2e. Scale bar: 1 cm. C. Live juveniles labeled for release into the lake.

The main problem encountered with the use of the cages (of both sizes) was the growth of calcified algae (probably a consequence of the hard water of Lake Banyoles); this blocked the mesh and isolated the mussels from the environment. This could be avoided by regular cage cleaning. This blocking was probably the reason why the *U. ravoisieri* juveniles grew better in the outdoor plastic channels (System 2b) than in the cages (System 2e) (Figure 4B) ($F=93.7$, $p<0.001$). At 2 or 3 years of age, the mussels raised in cages are mature enough to be released into the wild.

The survival of the juveniles freely seeded in the lake (System 2f) remains to be fully studied, but preliminary observations (June 2014) suggest that they are still alive. The release of infected fish was also shown to be a successful way of seeding the environment, at least in the lake effluents. This could be an easy and effective means of repopulating depleted areas but it would also be very difficult to monitor, and success might vary between one water system and another.

The results obtained with the indoor plastic trays (System 1) were not as good as those obtained with the pools (System 2 and its subsystems); survival and growth were much slower (Figure 3) ($F=66.89$, $p<0.001$). However, these trays provide an easy way of maintaining juveniles for study and handling in the laboratory.

In the experiment in which extra food was added (which was very laborious), the growth rates were much lower than those recorded for the other systems, although some juveniles did survive for more than one year (Figure 5).

The fish populations of Lake Banyoles have completely changed over the last century. The five native fish species (*Anguilla anguilla, Gasterosteus aculeatus, Barbus meridionalis, Squalius laietanus, and Salaria fluviatilis*) have been partially eliminated and replaced by the exotic *Micropterus salmoides, Lepomis gibbosus, Cyprinus carpio, Perca fluviatilis* and *Sander luciperca* (Moreno-Amich et al., 2006). This led to the vanishing of the formerly abundant populations of *U. mancus* and *U. ravoisieri*.

Restocking with native fish and naiads using the present systems is vital for the survival of these endangered molluscs in the lake.

In recent years, our knowledge of the reproductive biology of several *Unio* species has greatly increased (Aldridge and Mcivor, 2003; Araujo et al., 2005; Vincentini, Araujo et al., 2009b; Reis et al., 2013); this information could be used to better conserve these endangered freshwater mussels. The present results increase our knowledge of the reproductive strategy of *U. mancus* and *U. ravoisieri*. Several species of fish have been reported as successful hosts for the production of *U. mancus*

juveniles (Araujo et al., 2005), to which *B. meridionalis* and *T. tinca* (results not shown) can now be added. In *U. ravoisieri*, the breeding season of which was unknown, we now have shown that glochidia are released between April and July, and *B. meridionalis, S. laietanus, L. graellsi, S. fluviatilis, T. tinca* and *P. phoxinus* may be valid hosts, as shown in this work and preliminary testing.

Although much remains to be learned regarding the diet of freshwater mussels, the present results show that some species can be cultured in semi-captivity using only the water and sediment from their natural eeosystem. The survival rate of these juveniles in the following years will give a better idea about the viability of these breeding systems.

ACKNOWLEDGMENTS

This work was funded by the LIFE 08 NAT/E/000078 "Estany Project". The authors thank Ramón Casadevall and Arnau Juscafresa for their help in capturing the fish used in this work.

LITERATURE CITED

Aldridge, D.C. and A.L. Mcivor. 2003. Gill evacuation and release of glochidia by *Unio pictorum* and *Unio tumidus* (Bivalvia: Unionidae) under thermal and hypoxic stress. Journal of Mollusean Studies 69: 55–59.

Anonymous. 1914. The story of my life by "Billie Button". Wisconsin Pearl Button Co., 14 pp.

Araujo, R., N. Cámara, and M.A. Ramos. 2002. Glochidium metamorphosis in the endangered freshwater mussel *Margaritifera auricularia* (Spengler, 1793): A histological and scanning electron microscopy study. Journal of Morphology 254: 259–265.

Araujo, R., M. Quirós, and M.A. Ramos. 2003. Laboratory propagation and culture of juveniles of the endangered freshwater mussel *Margaritifera auricularia* (Spengler, 1793). Journal of Conchology 38: 53–60.

Araujo, R., I. Gómez, and A. Machordom. 2005. The identity and biology of *Unio mancus* (= *U. elongatulus*) (Bivalvia: Unionidae) in the Iberian Peninsula. Journal of Molluscan Studies 71: 25–31.

Araujo, R., J. Reis, A. Machordom, C. Toledo, M.J. Madeira, I. Gómez, J.C. Velasco, J. Morales, J.M. Barea, P. Ondina, and I. Ayala. 2009a. Las náyades de la península Ibérica. Iberus 27: 7–72.

Araujo, R., C. Toledo, and A. Machordom. 2009b. Redescription of *Unio gibbus* Spengler, 1793, a west palaearctic freshwater mussel with hookless glochidia. Malacologia 51: 131–141.

Barnhart, M.C. 2006. Buckets of muckets: A compact system for rearing juvenile freshwater mussels. Aquaculture 254: 227–233.

Barnhart M.C., W.R. Haag, W.N. Roston. 2008. Adaptations to host infection and larval parasitism in Unionoida. Journal of the North American Benthological Society 27: 370–394.

Beck, K. and R.J. Neves. 2003. An evaluation of selective feeding by three age-groups of the Rainbow mussel *Villosa iris*. North American Journal of Aquaculture 65: 203–209.

Claassen, C. 1994. Washboards, pigtoes and muckets: historie musseling in the Mississippi watershed. Historical Archeology 28: 1–145.

Coker, R.E., A.F. Shira, H.W. Clark, and A.D. Howard. 1921. Natural history and propagation of fresh-water mussels. Bulletin of the United States Bureau of Fisheries 37: 77–181.

Comas, O. and N. Valls. 2007. Informe de censos, seguiment i cria en captivitat de cranc de riu autòcton (Austrapotamobius pallipes) i d' Unio elongatulus aleroni a les rieres de Merlès, Gavarresa, Lluçanesa i Segalers. Generalitat de Catalunya, Departament de Medi Ambient i Habitatge, 79 pp.

Cuttelod, A., M. Seddon, and E. Neubert, E. 2011. European Red List of Non-marine Molluscs. Publieations Office of the European Union, Luxembourg.

Eversole, A.G., K.R. Stuart, and D.E. Brume. 2008. Effect of temperature and phytoplankton concentration of partitioned aquaculture system water on freshwater mussel filtration. Aquaculture Research 39: 1691–1696.

Eybe, T., F. Thielen, T. Bohn, and B. Sures. 2013. The first millimetre-rearing juvenile freshwater pearl mussels (*Margaritifera margaritifera* L.) in plastic boxes. Aquatic Conservation: Marine and Freshwater Ecosystems 23: 964–975.

Gatenby, C.M., R.J. Neves, and B.C. Parker. 1996. Influence of sediment and algal food on cultured juvenile freshwater mussels. Journal of the North American Benthological Society 15: 597–609.

Gatenby, C.M., D.M. Orcutt, D.A. Kreeger, B.C. Parker, V.A. Jones, and R.J. Neves. 2003. Biochemical composition of three algal species proposed as food for captive freshwater mussels. Journal of Applied Phycology 15: 1–11.

Gatenby, C.M., B.C. Parker, and R.J. Neves. 1997. Growth and survival of juvenile rainbow mussels, *Villosa iris* (Lea, 1829) (Bivalvia: Unionidae), reared on algal diets and sediment. American Malacological Bulletin 14: 57–66.

Geist J. 2010. Strategies for the conservation of endangered freshwater pearl mussels (*Margaritifera margaritifera* L.): a synthesis of conservation genetics and ecology. Hydrobiologia 644: 69–88.

Gum, B., M. Lange, and J. Geist. 2011. A critical reflection on the success of rearing and culturing juvenile freshwater mussels with a focus on the endangered freshwater pearl mussel (*Margaritifera margaritifera* L.). Aquatic Conservation: Marine and Freshwater Ecosystems 21: 743–751.

Guyot, J.A. 2005. Restoration of the endangered Cumberland elktoe (Alasmidonta atropurpurea) and Cumberland bean (Villosa trabalis) (Bivalvia: Unionidae) in the Big South Fork National River and Recreation Area, Tennessee and Kentucky. Master of Science Thesis, Virginia Polytechnic Institute and State University, 146 pp.

Henley, W.F., L. Zimmerman, and R.J. Neves. 2001. Design and Evaluation of Recirculating Water Systems for Maintenance and Propagation of Freshwater Mussels. North American Journal of Aquaculture 63: 144–155.

Howard, A.D. 1922. Experiments in the culture of freshwater mussels. Bulletin o the U. S. Bureau of Fisheries 38: 63–89.

Hruska, J. 1999. Nahrungsansprüche der Flussperlmuschel und deren halbnatürliche Aufzucht in der Tschechischen Republik. Heldia 4: 69–79.

Hudson, R.G. and B.G. Isom. 1984. Rearing juveniles of the freshwater mussels (Unionidae) in a laboratory setting. The Nautilus 98: 129–135.

Jones, J.W., R. Mair, and R. Neves. 2005. Factors Affecting Survival and Growth of Juvenile Freshwater Mussels Cultured in Recirculating Aquaculture Systems. North American Journal of Aquaculture 67: 210–220.

Kat, P.W. 1984: Parasitism and the Unionacea (Bivalvia). Biological Review 59: 189–207.

Khalloufi, N., C. Toledo, A. Machordom, M. Boumaïza, and R. Araujo. 2011. The unionids of Tunisia: taxonomy and phylogenetic relationships, with redescription of *Unio ravoisiseri* Deshayes, 1847 and *U. durieui* Deshayes, 1847. Journal of Molluscan Studies 77: 1–13.

Kovitvadhi, S., U. Kovitvadhi, P. Sawangwong, and J. Machado. 2008. A laboratory-scale recirculating aquaculture system for juveniles of freshwater pearl mussel *Hyriopsis (Limnoscapha) myersiana* (Lea, 1856). Aquaculture 275: 169–177.

Kovitvadhi, S., U. Kovitvadhi, P. Sawangwong, A. Thongpan, and J. Machado, J. 2006. Optimization of diet and culture environment for larvae and juvenile freshwater pearl mussels, *Hyriopsis (Limnoscapha) myersiana* Lea, 1856. Invertebrate Reproduction and Development 49: 61–70.

Lefevre, G. and W.C. Curtis. 1912. Studies on the reproduction and artificial propagation of fresh-water mussels. Bulletin of the Bureau of Fisheries 30: 105–201.

Liberty, A. 2004. An Evaluation of the Survival and Growth of Juvenile and Adult Freshwater Mussels at the Aquatic Wildlife Conservation Center (AWCC), Marion, Virginia. Master of Science Thesis, Virginia Polytechnic Institute and State University, 151 pp.

Liberty, A., B.J. Ostby, and R. Neves. 2007. Determining a suitable size and sampling frequency for rearing juvenile Rainbow mussels *Villosa iris*. North American Journal of Aquaculture 69: 44–52.

Lydeard, C., R.H. Cowie, W.F. Ponder, A.E. Bogan, P. Bouchet, S.A. Clark, K.S. Cummings, T.J. Frest, O. Gargominy, D.G. Herbert, R. Hershler, K.E. Perez, B. Roth, M. Seddon, E.E. Strong, and F.G. Thompson. 2004. The global decline of nonmarine mollusks. BioScience 54: 321–330.

Moreno-Amieh, R., Q. Pou-Rovira, A. Vila-Gispert, L. Zamora, and E. Garcia-Berthou. 2006. Fish ecology in Lake Banyoles (NE Spain): a tribute to Ramon Margalef. Limnetica 25: 321–334.

Nichols, S.J. and D. Garling. 2000. Food-web dynamics and trophic-level interactions in a multispecies community of freshwater unionids. Canadian Journal of Zoology 78: 871–882.

Nichols, S.J. and D. Garling. 2002. Evaluation of substitute diets for live algae in the captive maintenance of adult and subadult unionidae. Journal of Shellfish Research 21: 875–881.

O'Beirn, F.X., R.J. Neves, and M.B. Steg. 1998. Survival and growth of juvenile freshwater mussels (Unionidae) in a recirculating aquaculture system. American Malacological Bulletin 14: 165–171.

Prié, V. and N. Puillandre. 2013. Molecular phylogeny, taxonomy, and distribution of French *Unio* species (Bivalvia, Unionidae). Hydrobiologia 735: 95–110.

Reis, J., M.J. Collares-Pereira, and R. Araujo. 2013. Host specificity and metamorphosis of the glochidium of the freshwater mussel *Unio tumidiformis* (Bivalvia: Unionoidea). Folia parasitologica 61: 81–89.

Rogers-Lowery C.L. and R.V. Dimoek, Jr. 2006: Encapsulation of attached ectoparasitic glochidia larvae of freshwater mussels by epithelial tissue on fins of naïve and resistant host fish. Biological Bulletin 210: 51–63.

Strayer, D. L., J.A. Downing, W.R. Haag, T.L. King, J.B. Layzer, T.J. Newton, and S.J. Nichols. 2004. Changing perspectives on pearly mussels, North America's most imperiled animals. BioScience 54: 429–439.

Vincentini, H. 2005. Unusual spurting behaviour of the freshwater mussel *Unio crassus*. Journal of Molluscan Studies 71: 409–410.

Wächtler, K., M.C. Dreher-Mansur, and T. Richter. 2001. Larval types and early postlarval biology in naiads (Unionoida). In: *Ecology and Evolution of the freshwater mussels Unionoida.* (G. Bauer and K. Wächtler, eds.), pp. 93–125. Springer-Verlag, Berlin Heidelberg, Ecological Studies, 145.

Young, M. and J. Williams. 1984. The reproductive biology of the freshwater pearl mussel *Margaritifera margaritifera* (Linn.) in Scotland. I. Field studies. Archiv für Hydrobiologie 99: 405–422.